# The zebrafish forkhead transcription factor FoxH1/Fast1 is a modulator of Nodal signaling required for organizer formation

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**Background:** Signaling molecules related to the Nodal protein play essential roles in the formation and patterning of the gastrula organizer and the germ layers during vertebrate development. The forkhead transcription factor FoxH1 (also known as Fast1) is a component of the Nodal signaling pathway. Although different roles have been suggested for FoxH1, its specific function during development is still unclear.

**Results:** We report that the zebrafish locus *schmalspur* (*sur*) encodes a member of the FoxH1 family. Maternal *sur* transcripts were localized to the animal pole during oogenesis. Further expression was detected in a dorsoventral gradient at the onset of gastrulation and in specific domains in the organizer, notochord and lateral plate mesoderm. Embryos lacking zygotic *sur* function had variable deficiencies of prechordal plate and ventral neuroectoderm. In the absence of both maternal and zygotic *sur* function, embryos failed to form a morphologically distinct gastrula organizer and, later, developed severe defects in all axial structures. In these embryos, expression of *nodal* genes was initiated but not maintained. Unlike embryos lacking Nodal signaling, *sur* mutants formed endoderm and paraxial mesoderm.

**Conclusions:** FoxH1 is involved in regulatory feedback loops that control the duration and intensity of Nodal signals in early patterning. In zebrafish, FoxH1 is not essential to induce Nodal-dependent cell fates, but its function is central in modulating and enhancing morphogenetic Nodal signals.

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## Background

A fundamental goal of developmental biology is to understand the mechanisms that establish the vertebrate body plan. Among the variety of signaling molecules identified, two subgroups of the transforming growth factor-β (TGF-β) superfamily have been shown to provide key morphogenic signals during the early patterning processes in the vertebrate embryo. Bone morphogenic proteins (BMPs) pattern the gastrula embryo along the dorsoventral axis and are required to define ventral cell fates. Nodal-related proteins are required for the formation of the gastrula organizer, induction of mesoderm, and specification of the left–right axis.

Nodal was originally identified in a screen for retroviral integrations affecting mouse development [1]. Mouse embryos that are homozygous mutant for the *nodal* gene arrest in gastrulation and die early in development. More detailed analyses revealed primary functions of Nodal in the formation and maintenance of the primitive streak and patterning of anterior neural structures [2,3]. In zebrafish, two *nodal*-related genes — *squint* (*sqt*) and *cyclops* (*cyc*) — are required for the formation of the organizer, endoderm and trunk mesoderm [4,5]. Because of the overlapping expression and the similar activities of *cyc* and *sqt*, embryos that are homozygous mutant for either *cyc* or *sqt* show only partial loss of axial mesoderm and ventral neuroectoderm, whereas *cyc/sqt* double mutants lack most mesendodermal tissues. In *Xenopus*, four *nodal*-related genes — *xnr1–xnr4* — have been identified [6–8]. Consistent with a conserved role of Nodal signaling in dorsal mesodermal induction, these genes are expressed in dorsal marginal cells, which underlie the prospective dorsal mesoderm, and overexpression of *xnr1*, *xnr2* and *xnr4* in animal caps induces mesodermal differentiation [6,8,9].

Overexpression experiments in *Xenopus* and zebrafish show that lower levels of Nodal signaling induce expression of the pan-mesodermal marker *brachyury* or the notochord marker *floating head* (*flh*), whereas higher doses induce the prechordal plate marker *goosecoid* (*gsc*) [6,10]. These data indicate that Nodal signals have additional morphogenic activities. Recent studies in *Xenopus* have demonstrated that Nodal-related proteins are involved in mesodermal patterning [9,11]. These studies are based on overexpression of a *Xenopus* Lefty orthologue, which blocks Nodal signaling at the level of the receptor, or of a truncated form of Cerberus, which binds directly and inactivates

Nodal-related proteins. In zebrafish, overexpression of the Lefty orthologue Antivin blocks formation of endoderm and mesoderm, and at high concentration also blocks the formation of posterior neuroectoderm [12]. Together, these results suggest that Nodal-related proteins provide morphogenic signals that pattern the pregastrula embryo along the animal–vegetal axis [13].

The molecular events underlying the transduction of the distinct Nodal-related signals are just now beginning to be understood [13,14]. Genetic and embryological analysis of the *one-eyed pinhead* (*oep*) locus in zebrafish identified the extracellular membrane-associated EGF-CFC protein as an essential mediator of Nodal, but not Activin, signals [15,16]. Embryos that lack maternal and zygotic *oep* in zebrafish, or that lack the orthologue *cripto* in the mouse, develop phenotypes very similar to the corresponding *nodal* mutants [16–18]. Among the intracellular signal transducers, Smad2 and Smad4 have been shown to be essential mediators of Nodal signals in the mouse. After ligand-stimulated activation of Activin/Nodal receptors, Smad2 and possibly Smad3 are phosphorylated, form a multimeric complex with Smad4, and translocate to the nucleus. In the nucleus, the complexes associates with different DNA-binding proteins, including the forkhead transcription factor FoxH1 (also known as Fast1; for nomenclature, see [19]) and paired-class homeobox proteins of the Mix/Bix family [20] . Although it is not clear whether the Smad complexes alone are sufficient to activate transcription of target genes, promotor studies in transient reporter assays and in transgenic mice have indicated essential roles of FoxH1 for the transmission of Nodal signals. FoxH1-binding sites that are found in the regulatory regions of several genes including *mix.2*, *gsc*, *bhikhari* as well as *nodal*- and *lefty*-related genes have been shown to be required for full activation of the respective reporter construct [21–27]. Together with studies in *Xenopus* that use a dominant-negative form of *Xenopus* FoxH1, or antibodies that specifically block FoxH1 function, these data suggest that FoxH1 is the endogenous mediator of inductive Nodal signals [27,28]. The developmental role of FoxH1 and its function in morphogenic Nodal signaling remain unclear, however.

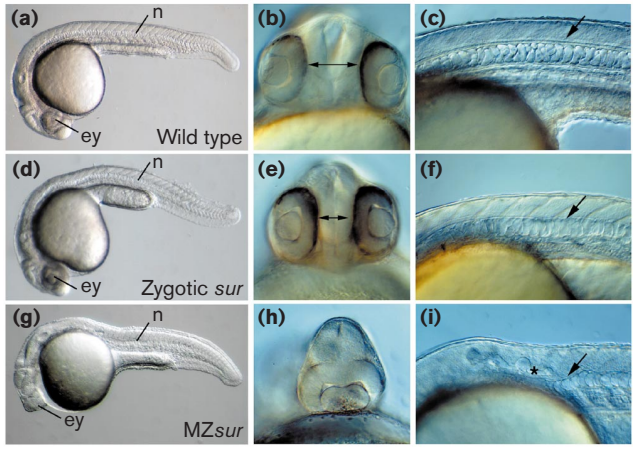
Here, we report that the zebrafish locus *schmalspur* (*sur*) encodes an orthologue of FoxH1. Zebrafish FoxH1 is expressed maternally and zygotically and, consistent with its expression, has both maternal and zygotic functions in development. Our studies confirm that FoxH1 is a conserved component of the Nodal signaling pathway. Unlike the suggested role of FoxH1 in *Xenopus*, our experiments revealed that FoxH1 is not strictly required for induction of Nodal-dependent cell fates in zebrafish. Instead, we found that FoxH1 is primarily required in an autoregulatory feedback loop that modulates and enhances morphogenic Nodal signals.

## Results and discussion

### Maternal and zygotic functions of *schmalspur* in zebrafish development

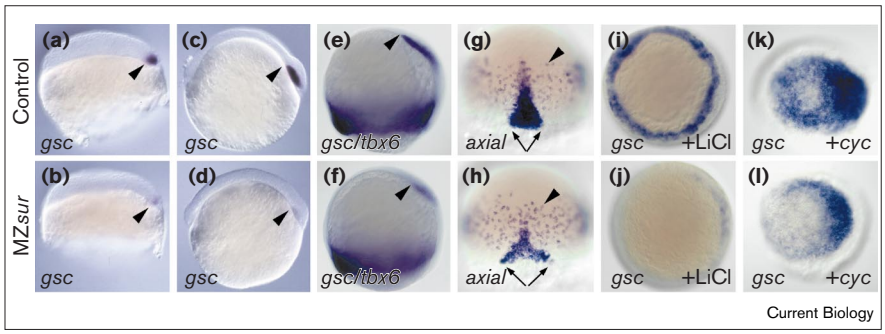
The zebrafish mutations *schmalspur* (*sur*$^{ty68b}$) and *uncle freddy* (*unf*$^{m768}$) have been independently described as recessive, embryonic lethal mutations that result in similar phenotypes [29,30]. At 24 hours post fertilization (24 hpf), the phenotype includes ventral body curvature, absence or reduction of floorplate, reduced prechordal plate, and synopthalmia (Figure 1d–f). Our complementation analysis revealed a similar phenotype for transheterozygous embryos, indicating that *unf*$^{m768}$ and *sur*$^{ty68b}$ are allelic. Hence, the locus will be referred to as *sur*. Penetrance and expressivity of the *sur* mutant phenotype strongly depended on the genetic background and were variable even within a single clutch of embryos. The variable expressivity allowed us to generate fertile homozygous *sur*$^{m768/m768}$ fish. In crosses of homozygous mutant males and heterozygous females, 10–50% of the embryos developed phenotypes as those described above. In contrast, crosses of homozygous females with heterozygous males produced 50% mutant embryos that developed more severe patterning defects, revealing a maternal contribution of *sur*. Maternal and zygotic *sur* (MZ*sur*) mutant embryos showed a strong

**Figure 1**




Maternal and zygotic function of *sur* in axis formation. **(a–c)** Wild-type, **(d–f)** zygotic *sur*$^{m768}$ and **(g–i)** MZ*sur*$^{m768}$ embryos at 27 hpf. The eyes (ey, arrows indicate the spacing of the eyes), notochord (n) and floorplate (arrow) are indicated. Embryos lacking zygotic *sur* developed (d) ventral body curvature, (e) reduced spacing of the eyes, and (f) lacked ventral neuroectoderm including the floorplate, visible in the wild type as a fine line dorsal to the notochord. MZ*sur* mutant embryos developed (g) anterior truncations of the brain and, frequently, a shortened body axis, (h) cyclopia (50%) or variable synopthalmia. (i) Frequently, ectopic notochord cells (asterisk) formed within the neural tube. Embryos are shown from a lateral view with dorsal uppermost and anterior to the left in (a,b,d,e,g,h), or from an anterior view with dorsal uppermost in (c,f,i). The middle and right columns show, at higher magnification, the embryos depicted in the left column.

**Figure 2**




*Sur* is required for proper organizer formation. Expression of organizer genes in control (upper row) and MZ*sur* (lower row) embryos. **(a–f)** Comparison of *gsc* expression in (a,b) marginal dorsal cells (arrowhead) at 30% epiboly, (c,d) involuting cells in the shield (arrowhead) at 60% epiboly, and (e,f) prechordal plate mesoderm (arrowhead) and overlying neuroectoderm at 90% epiboly revealed a strongly reduced number of *gsc*-expressing cells in MZ*sur* mutant embryos. Embryos in (e,f) were also stained for the paraxial mesoderm marker *tbx6*. Note the mesodermal expression of *tbx6* at 90% epiboly in MZ*sur* mutant embryos. **(g,h)** Expression of *axial* in endoderm (arrowhead) and dorsal midline (arrows) at 80% epiboly. Expression of *axial* in endoderm cells was normal, but a reduced number of *axial*-expressing cells were found in the midline; note the wider *axial* expression domain (arrows). **(i)** Ectopic expression of *gsc* at 50% epiboly in all marginal cells of wild-type embryos after LiCl treatment [31]; animal-pole view. **(j)** No induction of *gsc* could be found after LiCl treatment of MZ*sur* mutants. **(k,l)** Ectopic expression of *gsc* 8 h after injection of 1 pg *cyc* mRNA at the one-cell stage (animal view). Note the reduced levels of *gsc* expression in the MZ*sur* embryo compared with the control embryo. The genotypes of all embryos were confirmed by analysis of *sur*$^{m768}$-linked alleles of SSLP markers Z22103 and Z10806. Panels (a–f) are lateral views with dorsal on the right and animal pole at the top; (g,h) are dorsal views, with the animal pole at the top; (i–l) animal-pole view with dorsal on the right.

reduction of the prechordal plate and floorplate, ventral brain defects, strong synopthalmia or cyclopia, and notochord abnormalities (Figure 1g–i). Heterozygous embryos from these crosses appeared wild type (control embryos), indicating that maternal *sur* function is not strictly required for normal development. Thus, the genetics of *sur* are very similar to those of the maternally acting and paternally rescuing *oep* locus [16].

### The *sur* gene is required in dorsal specification and organizer formation

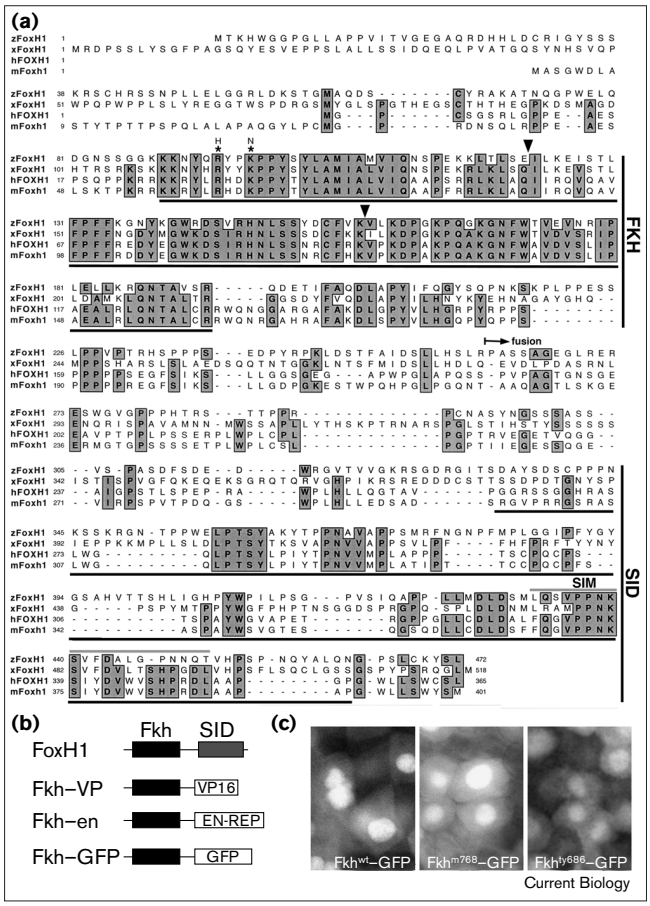
MZ*sur* mutant embryos could first be identified morphologically at early gastrula stages by the absence or reduced size of the embryonic shield, the zebrafish equivalent of the Spemann gastrula organizer. Expression analysis of several organizer-specific genes in late blastula MZ*sur* mutants revealed a strong reduction of *gsc* [31] expression (Figure 2a–f). The loss of prechordal mesoderm in MZ*sur* mutants corresponded to the lack of early *gsc* expression [10]. Expression domains of *chordin* [32], and *bozozok* (also known as *dharma* or *nieuwkoid*) [33–35] appeared normal, but expression of *flh* [36] was affected to a variable degree (data not shown). In embryos lacking either maternal or zygotic *sur*, expression of *gsc* was normal or only slightly reduced, indicating redundant functions of maternal and zygotic *sur* during blastula stages. Together, these data suggest that *sur* functions in early dorsal specification and in organizer formation.

Studies of the *gsc* promotor in *Xenopus* and zebrafish have identified a β-catenin response element and at least one Activin-response element upstream of the transcription start site [20,37]. Studies of the zebrafish mutations *sqt*, *cyc* and *oep* have revealed that Nodal-related signals are required for induction and maintenance of early *gsc* expression [4,5,10,16,38,39]. To elucidate which of the molecular pathways regulating *gsc* expression require *sur* function, embryos were treated with the dorsalizing agent LiCl, or injected with *β-catenin*, *cyc* or *sqt* RNA. Exposure of early cleavage embryos to lithium ions promotes nuclear localization of β-catenin, leading to ectopic activation of *gsc* throughout the circumference of late blastula embryos [31] (Figure 2i). In MZ*sur* mutant embryos, LiCl treatment or injection with *β-catenin* RNA failed to upregulate expression of *gsc* (Figure 2j, data not shown). In contrast, injections of *cyc* or *sqt* RNA were sufficient to induce ectopic expression of *gsc* in MZ*sur* mutants (Figure 2k,l; data not shown). The results show that *sur* acts downstream or parallel to β-catenin and that the *sur* mutations do not prevent Nodal-dependant induction of *gsc*. Nevertheless, the reduced levels of *gsc* transcription in *cyc*- or *sqt*-injected MZ*sur* mutants compared with injected control embryos indicate an involvement of *sur* in Nodal signaling.

### The *sur* locus encodes a novel FoxH1 protein

To clone the *sur* gene, we mapped *sur*$^{m768}$ to the centromere of linkage group 12 and identified tightly linked genetic markers. In a chromosomal walk, we were able to cover the critical region with genomic yeast artificial chromosome (YAC) clones. Radiation-hybrid mapping [40–42] of candidates revealed no recombination between the closest simple-sequence length polymorphism (SSLP) marker Z22103 and a FoxH1 [19] orthologue available in the expressed sequence tag (EST) database at the National Center for Biotechnology Information (NCBI). To further analyze the *foxH1* gene, we isolated the full-length coding

**Figure 3**




The *sur* locus encodes FoxHI. **(a)** Alignment of predicted FoxH1 proteins from zebrafish (zFoxH1), *Xenopus* (xFoxH1), human (hFOXH1) and mouse (mFoxh1). Gray shading, conserved amino acids; asterisks, point mutations in $sur^{m768}$ (CGT→CAT, $R^{94}$→$H^{94}$) and $sur^{ty68b}$ (AAA→AAT, $K^{97}$→$N^{97}$); arrowheads, positions of introns in the corresponding genomic DNA; gray line, the Smad-interaction motif (SIM; also found in Mix/Bix proteins). The FKH domain and SID are underlined. The position of the cloning site used to generate fusion proteins is marked (arrow, fusion). **(b)** Schematic illustration of the fusions between the FKH domain of wild-type or mutant FoxH1 and GFP (Fkh–GFP), the Engrailed repressor domain (EN-REP; Fkh–en), or VP16 (Fkh–VP). **(c)** Nuclear localization of the wild-type and mutant Fkh–GFP proteins in 60% epiboly embryos after injection of 50 pg RNA encoding FKH–GFP. Nuclear levels of GFP fluorescence were similar for $Fkh^{m768}$–GFP and $Fkh^{wt}$–GFP and slightly reduced for $Fkh^{ty68b}$–GFP.

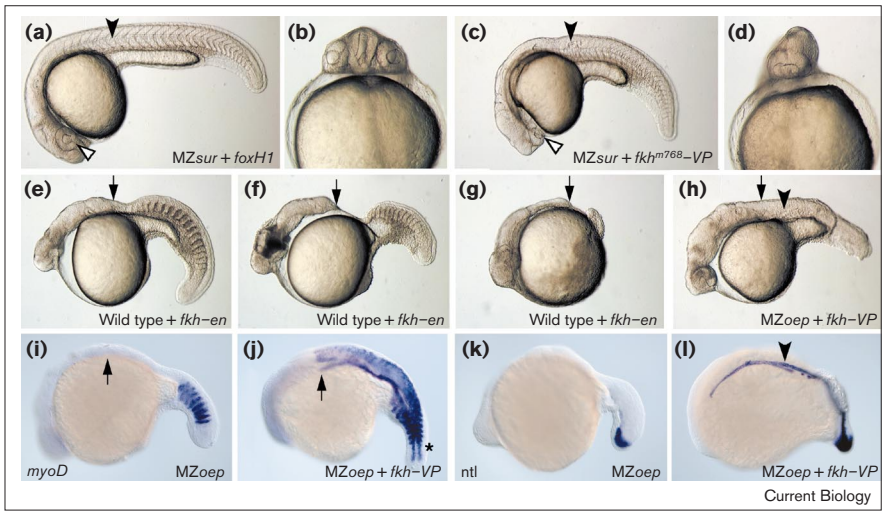
region from genomic and cDNA. Sequencing of the wild-type cDNA revealed a putative open reading frame of 1426 bp, which encoded a protein with highest sequence similarity to *Xenopus* FoxH1 (Figure 3a). The overall homology to known FoxH1 proteins was low (27–34% identity) but similar to that between mammalian and *Xenopus* FoxH1 [43–46]. The highest homology is found in the forkhead domain (FKH, 59–77% identity), and in segments of the carboxy-terminal Smad-interaction domain (SID) that have previously been shown to mediate interaction with activated Smad2 and Smad4 proteins [20,47] (Figure 3a). Sequence comparison of *foxH1* cDNA from wild-type and *sur* mutant embryos revealed distinct missense mutations in $sur^{m768}$ and $sur^{ty68b}$. Both mutations altered conserved amino acids ($R^{94}$→$H^{94}$ and $K^{97}$→$N^{97}$; Figure 3a, asterisks) within the amino-terminal part of the FKH domain, which has been connected with DNA binding and nuclear localization [48]. Injections of 10–40 pg wild-type, but not the mutant, zebrafish *foxH1* mRNA was sufficient to rescue the *sur* mutant defects (Figure 4a,b; data not shown). Injections of a higher concentration of wild-type FoxH1 resulted in deformed embryos (data not shown). Together, these data provide compelling evidence that *sur* encodes the FoxH1 protein and they suggest that *sur* mutant FoxH1 proteins are completely inactive.

*In situ* expression analysis revealed that *foxH1* mRNA is expressed maternally and zygotically. During oogenesis, *foxH1* was detected at all stages analyzed, with the transcripts confined to the prospective animal pole early in oogenesis (Figure 5a,b). A similar mRNA localization has been described for other genes potentially involved in TGF-β signaling (*vg1* [49], *vg1-rbp* [50] and *taram-A* [49]). After fertilization, zebrafish *foxH1* transcripts were evenly distributed until late blastula stages (Figure 5c). At the beginning of gastrulation, *foxH1* mRNA was expressed in a ventral to dorsal gradient, with highest levels on the ventral and low levels on the dorsal side but also with high levels in the shield (Figure 5d–f). During gastrulation, *foxH1* expression became progressively restricted to midline and ventral cells (Figure 5g–i). At the onset of somitogenesis, *foxH1* transcripts were detected exclusively in notochord, lateral plate mesoderm and in a stripe of anterior dorsal neuroectoderm (Figure 5j–l). Staining in these tissues persisted during somitogenesis but no transcripts could be detected after 27 hpf.

### Mesendoderm-inducing activities of wild-type and mutant FoxH1

To determine how the *sur* mutations interfere with FoxH1 function and to study the biological activities of FoxH1, we generated chimeric proteins of the wild-type or mutant FoxH1 FKH domain fused to either the transcriptional repressor domain of Engrailed (Fkh–en) or the viral transcriptional activator domain of VP16 (Fkh–VP; Figure 3b). Similar constructs have been made for *Xenopus* FoxH1 and were shown to work as efficient repressors or activators of *Xenopus* FoxH1 target genes [28]. Wild-type embryos injected with RNA encoding wild-type Fkh–en ($fkh^{wt}$–*en* RNA) developed phenotypes similar to M*Zoep* mutants or to embryos injected with RNA encoding the Nodal and Activin antagonist Antivin/Lefty1 (Atv; Figure 4e–g) [12,16,51]. The phenotypes ranged from partial loss of endoderm and axial mesoderm, to absence of trunk and tail structures. Conversely, overexpression of RNA encod-

**Figure 4**




Activities of FoxH1. **(a,b)** Phenotypic rescue of MZ*sur* mutants by injection of 10 pg *foxH1* RNA. (a) Lateral and (b) anterior view at higher magnification. **(c,d)** Injection of 100 pg *fkh$^{m768}$–VP* RNA had no effect on MZ*sur* mutant development (compare with Figure 1). (c) Lateral and (d) anterior view. Unshaded arrowheads indicate the eye, and shaded arrowheads the notochord. **(e–g)** Wild-type embryos injected with 10 pg *fkh–en* RNA developed variable patterning defects: (e) severe midline defects, synopthalmia or cyclopia (14/37 injected embryos); (f) a phenotype similar to MZ*oep* mutants, including lack of trunk mesoderm and cyclopia (21/37 injected embryos); and (g) narrowed hindbrain lacking trunk and tail structures (2/37 injected embryos). Arrows mark the head–trunk boundary. **(h–l)** Injection of 5 pg *fkh–VP* RNA partially rescued formation of somitic mesoderm (arrow) and notochord (arrowhead) in MZ*oep* mutants. (i,j) Rescued muscle-specific expression of *myoD* in trunk mesoderm and adaxial cells of the tail (asterisk). (k,l) Expression of *ntl* in MZ*oep* mutants.

ing wild-type Fkh–VP (*fkh$^{wt}$-VP* RNA) was sufficient to rescue formation of notochord, adaxial muscles and trunk mesoderm and in MZ*oep* mutants, as illustrated by expression of *myoD* and *no tail* (*ntl*; Figure 4g–l). Injection of the *sur*$^{m768}$ and *sur*$^{ty68b}$ mutant versions (*fkh$^{m768}$–VP* and *fkh$^{m768}$–en*) resulted in no effect on wild-type or *sur* mutant development even when we injected 20 times the amount of mRNA that was sufficient to induce abnormal development using *fkh$^{wt}$–VP* (Figure 4c,d). Thus, both mutations appeared to abolish the function of the FKH domain.

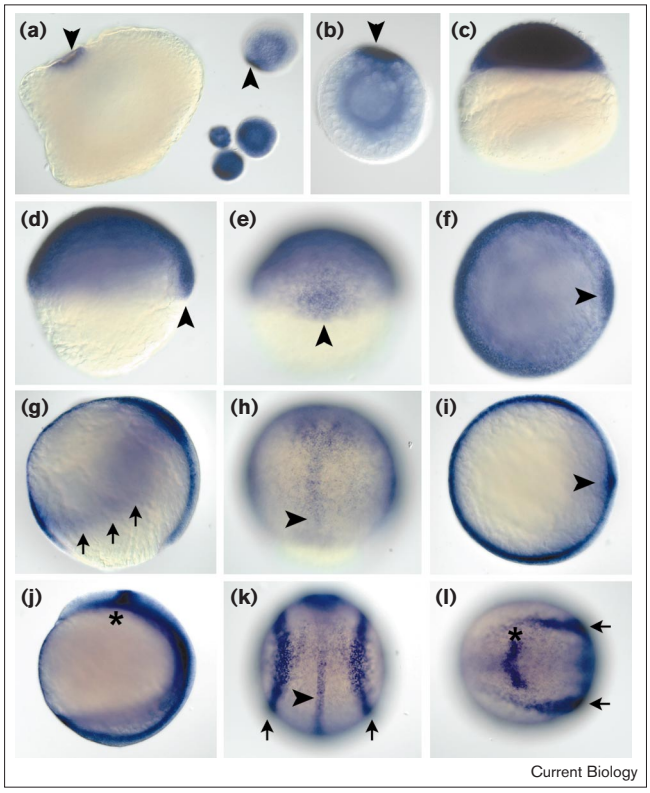
Fusion proteins of the wild-type or mutant FoxH1 FKH domain with the green fluorescent protein (Fkh–GFP) all exhibited nuclear localization, indicating that the mutations impair DNA binding rather than nuclear translocation of FoxH1 (Figure 3b,c). The carboxy-terminal part of the mutant proteins, including the SID, remained intact. We cannot exclude the possibility that mutant FoxH1, in complex with activated Smads and other potential binding partners, could still interact with DNA target sites to provide weak gene activation. Such an activity is unlikely, as overexpression of *Xenopus* SID in *Xenopus* and zebrafish blocks Nodal signaling instead of activating it [27,28]. Together with the inability of mutant FoxH1 proteins to rescue the *sur* phenotype, our results indicate a total loss of FoxH1 activity in *sur* mutants.

Embryos injected with *fkh–en* RNA developed with dose-dependent deficiencies in, or loss of, dorsal mesoderm and endoderm, as revealed by changes in the expression of *gsc*, *flh* and *axial* (Figure 6c,f,h) [38,52]. In contrast, *fkh–VP* RNA injection was sufficient to induce ectopic expression of *flh* and *gsc* in marginal cells of wild-type embryos (Figure 6b,e) and to rescue mesodermal and endodermal expression of *gata5* and *sox17* in MZ*oep* mutants (Figure 6i–l) [53,54]. These results support the idea that FoxH1 is involved in endoderm and mesoderm induction [28]. Our phenotypic analysis of MZ*sur* mutants revealed only minor defects in formation of endoderm and mesoderm derivatives and only a partial loss of axial mesoderm (Figure 2). Currently, we cannot exclude the existence of a second *FoxH* gene in zebrafish that could partly rescue loss of FoxH1 activity. Nevertheless, the insensitivity of *gsc* expression to ectopic β-catenin in MZ*sur* mutants (Figure 2i,j) suggests a total loss of FoxH1-related functions in these embryos. The different results of the overexpression and the genetic approach could therefore result from antimorphic rather than dominant-negative activities of *fkh–en*. Thus, we suggest that FoxH1 is involved in, but not required for, the induction of endoderm and mesoderm.

### FoxH1 is required for maintained expression of *nodal* genes

Genetic and embryological studies in zebrafish, *Xenopus*, and mouse have demonstrated essential roles of Nodal-related signals for the formation of mesoderm, endoderm and the gastrula organizer [13,55]. To determine whether FoxH1 is involved in Nodal signaling and, if so, at what level of the pathway it might act, we examined the expression patterns of *cyc*, *sqt* and *atv* [51,56] in *sur* mutant embryos. Expression of *cyc*, *sqt* and *atv* was initiated normally

**Figure 5**



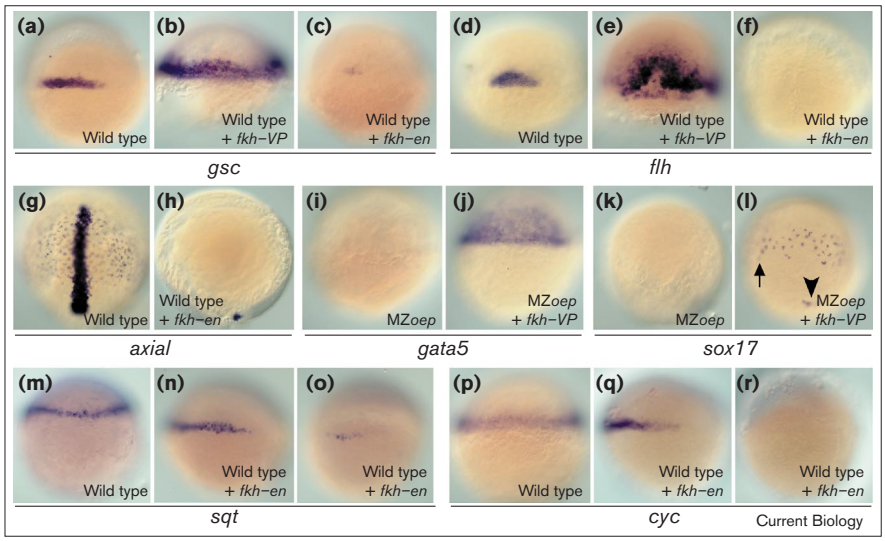

Expression of FoxH1 in oogenesis and early embryogenesis. **(a)** Maternal expression was first broad (right bottom) but became localized to the animal pole early in oogenesis (arrowheads). **(b)** Higher magnification of stage II oocyte. **(c)** Ubiquitous expression at sphere stage. **(d–f)** Expression in the shield (arrowhead) and in a ventral–dorsal gradient at 60% epiboly. **(g–i)** Expression in axial (arrowhead) and lateral mesoderm (arrows) at 90% epiboly. **(j–l)** Expression in notochord (arrowhead), lateral mesoderm (arrows) and brain (asterisk) at the one-somite stage. (d,g,j) Lateral, (e,h,k) dorsal and (f,i,l) anterior views.

in MZ*sur* mutants, but not maintained at late blastula and early gastrula stages (Figure 7). In MZ*sur* mutants, expression of zygotic *sqt* appeared wild type at the oblong stage but was reduced at 40% epiboly and absent at 70% epiboly (Figure 7a–f). Similarly, *cyc* was induced in MZ*sur* mutants but, at 40% epiboly, the level of expression was reduced when compared with that of the heterozygous control embryo (Figure 7g,h). The midline-specific expression of *cyc* during early gastrulation was strongly reduced or absent in MZ*sur* mutants (Figure 7i–l). These results suggest a function of FoxH1 in regulating *nodal* gene expression. The strong phenotypes of embryos injected with *fkh–en* could therefore be a secondary consequence of downregulation of *cyc* and *sqt* rather than of mesendoderm-specific downstream genes only. Accordingly, expression analyses revealed that injection of *fkh–en* RNA is sufficient to block expression of *cyc* and *sqt* in late blastula embryos (Figure 6m–r).

Control of *nodal* expression involves autoregulatory feedback loops [11,26,56,57]. A positive loop is required to maintain *nodal* gene expression, and a negative loop, which is mediated through Lefty-related proteins, prevents excessive spreading of the Nodal signal. Consistent with this idea, *atv* expression mirrored that of *cyc* and *sqt* during normal zebrafish development [51,56]. In MZ*sur* mutant embryos (Figure 7m,n), *atv* transcripts could no longer be detected after 30% epiboly (data not shown). More strikingly, *atv* mRNA was also absent in late blastula and gastrula stages of zygotic *sur* mutant embryos (Figure 7o–r). In agreement with previous conclusions drawn from enhancer analysis, our studies implicate roles for FoxH1 in both regulatory loops [26]. FoxH1-binding sites have been shown to be required for the activation of reporter construct upstream of the translational start site and on the first exon of *nodal*- and *lefty*-related genes of mouse, *Xenopus* and ascidians [21,22,26,27]. Nevertheless, similar to results in *Xenopus*, overexpression of *fkh–VP* was not sufficient to achieve maintained expression of *nodal* genes and *atv* in MZ*oep* mutants [27] (data not shown). This indicates either that functionally important domains within the SID have been removed in Fkh–VP, or that additional factors act in parallel to FoxH1 downstream of Nodal signals. In comparison to MZ*oep* mutants, early expression of *nodal* but not *atv* persisted longer in MZ*sur* mutants, also indicating that factors other than FoxH1 are involved in mediating the positive feedback loop [57]. Beside the early function of FoxH1 in the pregastrula embryo, the enhancer studies concentrated on the role of FoxH1 in left–right axis specification [24,26,27,58]. Consistent with their conclusions, recent phenotypic analyses of zygotic *sur* mutants have demonstrated a role of *sur* in establishing asymmetric *cyc* expression [59]. Together, these studies demonstrate a conserved role of FoxH1 as regulators of *nodal* gene expression.

Our results reveal that FoxH1 is not strictly required to transmit inductive Nodal signals in zebrafish. Further analysis of *foxH1* mutations in other organisms will be necessary to clarify whether this is a mechanism that is specific for zebrafish. Nevertheless, the recent finding that Mix/Bix proteins function as mediators of Nodal signaling [20] suggests that our model might be valid generally. A subclass of these proteins was shown to form, upon induction by Activin, complexes that activate transcription of the Nodal target gene *gsc*. Interaction of these Mix/Bix proteins with activated Smads is mediated by a small peptide motif (SIM, Figure 3a) that is also found in FoxH1 proteins. Currently, the only known zebrafish member of the Mix/Bix family is Mixer, which is encoded by the *bonny and clyde* locus [54,60]; *mixer* mutants form organizer and organizer derivatives but develop endodermal defects. The complementary phenotypes of *mixer* and *foxH1* mutants thus reveal distinct but temporarily overlapping activities of the encoded proteins downstream in

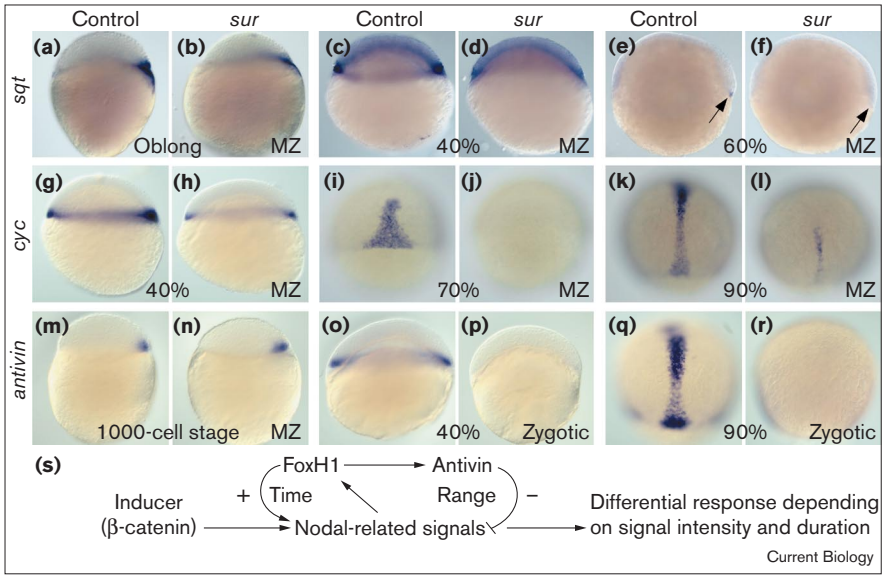
**Figure 6**




Activities of FoxH1 fusion proteins. **(a,d)** Expression of (a) *gsc* and (d) *flh* at 50% epiboly in wild-type embryos. **(b,e)** Ectopic marginal expression of (b) *gsc* (28/28 injected embryos) and (e) *flh* (12/12 injected embryos) after injection of 20 pg *fkh–VP* RNA. Loss of **(c)** *gsc* (12/28 injected embryos) and **(f)** *flh* (10/27 injected embryos) expression after injection of 20 pg *fkh–en* RNA. **(g)** Expression of *axial* at 90% epiboly in wild-type embryos. **(h)** Loss of *axial* expression in endoderm and axial mesoderm after injection of *fkh–en* RNA (10 pg: 5/31 injected embryos; 50 pg: 7/14 injected embryos). **(i,k)** In MZ*oep* mutants, there was no expression of (i) *gata5* at 50% epiboly or (k) *sox17* at 90% epiboly. **(j,l)** Rescued expression of (j) *gata5* (22/22 injected embryos) and (l) *sox17* (25/25 injected embryos) in MZ*oep* mutant embryos after injection of 20 pg *fkh–VP* RNA; note the expression of *sox17* in dorsal forerunner cells (arrowhead) and endodermal cells (arrow). **(m)** Expression of *sqt* in the margin of a wild-type embryo at 40% epiboly. **(n,o)** In embryos injected with 10 pg *fkh–en* RNA, *sqt* expression was reduced by (n) up to 50% (12/20 injected embryos) or (o) 50–100% (8/20 injected embryos). **(p)** Expression of *cyc* in a wild-type embryo at 40% epiboly. **(q,r)** In embryos injected with 50 pg *fkh–en* RNA, *cyc* expression was reduced by (q) up to 50% (13/19 injected embryos) or (r) 50–100% (6/19 injected embryos). All embryos are viewed from the dorsal side.

the Nodal signaling pathway. Whereas Mixer functions in early endoderm specification, FoxH1 ensures sustained presence of the highest Nodal signaling levels required to specify axial mesoderm in the organizer.

**Figure 7**




*Sur* is required for maintenance of *cyc*, *sqt* and *antivin* expression. **(a–f)** Lateral view of control and MZ*sur* mutant embryos (MZ), showing expression of *sqt* at (a,b) oblong stage; (c,d) 40% epiboly and (e,f) 60% epiboly. In MZ*sur* mutants, *sqt* expression was similar to the wild type at oblong stage, reduced at 40% epiboly and missing at 60% epiboly. **(g–l)** Dorsal view of control and MZ*sur* mutant embryos, showing expression of *cyc* at (g,h) 40% epiboly; (i,j) 70% epiboly and (k,l) 90% epiboly. In MZ*sur* mutants, *cyc* expression was reduced at 40% epiboly and missing or reduced at 70% and 90% epiboly. **(m–r)** Expression of *antivin* at (m,n) 1000-cell stage (lateral view), (o,p) 40% epiboly, and (q,r) 90% epiboly in (m,o,q,) control, (n) MZ*sur* mutant and (q,r) zygotic *sur* mutant embryos. Expression of *antivin* in *sur* mutant embryos was wild type at the 1000-cell stage but missing at 40% and 90% epiboly. **(s)** Model for the function of FoxH1 proteins (see also text): FoxH1 is a nuclear component of the Nodal signaling pathway that mediates autoregulatory feedback activation of *nodal* genes (+) and and extracellular negative feedback regulation of Nodal signals (–) by activating the Nodal antagonist Lefty. These activities make FoxH1 a key modulator of Nodal signaling, controlling time, range and intensity of the signals. Initial expression of *nodal* genes is independent of FoxH1 and involves pathways that also include β-catenin [9]. The arrows indicate the direction of signaling. Indirect interactions and additional regulation by other pathways or parallel Nodal-dependant signals may contribute to the interactions depicted.

## Conclusions

Recent studies in zebrafish and *Xenopus* have revealed morphogen-like activities of Nodal-related proteins that pattern the late blastula embryo along the animal–vegetal axis. To mediate these morphogenic activities, the expression of Nodal proteins requires tight regulation. Our results demonstrate that FoxH1 is an essential component of regulatory feedback loops required to maintain expression of *nodal* genes and *atv* in early development (autoregulatory positive and negative loops mediated by Lefty-related proteins; Figure 7s). Expression of FoxH1 in the organizer and in the notochord correlates with the high level of *cyc* expression in these tissues. Our data indicate a regulatory rather than an inductive role for FoxH1 in Nodal signaling. We therefore propose that FoxH proteins primarily function as modulators and local amplifiers of *nodal* gene expression, and thus determine exposure time, intensity and range of morphogenic Nodal signals (Figure 7s).

## Materials and methods

### *Zebrafish strains*

Adult fish and embryos were maintained as described [61]. Embryos were derived from matings of identified heterozygotes or homozygotes. The following mutant alleles were used: *sur*$^{m786}$ (*unf*$^{m768}$), *sur*$^{ty68b}$ and *oep*$^{m134}$ [29, 30]. Homozygote *oep* mutants were rescued to adulthood by RNA injection as described [16]. Homozygous *sur* mutants were generated from *sur*$^{m786/+}$ in-crosses (up to 50% of the homozygotes survived to adulthood) or by RNA rescue. Embryos were staged as described [62].

### *Genetic mapping*

A map cross between *sur*$^{m786/+}$ in the AB strain and India strain fish were used to generate a panel of >3000 *sur*$^{m768/m768}$ embryos. The closest linkage was found to the SSLP marker Z22103 (no recombinations). Radiation-hybrid mapping (Goodfellow T51 panel, Research Genetics) revealed no recombination between Z22103 and an EST with homology to FoxH1 (assembled sequence available as TC23065, TC21374: http://www.tigr.org/tdb/zgi/searching/reports.html). PCR primers were: Fas-s, 5′-CATATCGTGGAAAGGCCACT-3′; Fas-a, 5′-GAAGGTATG-GTCGCTCCTCA-3′. The EST was independently mapped by the group of W. Talbot http://zebrafish.stanford.edu/genome/zfishmap).

### *Isolation of genomic and cDNA of zebrafish* foxH1

FoxH1-encoding DNA was isolated by PCR (primers Fas-s: 5′-CAT-ATCGTGGAAAGGCCACT and Fas-2a: 5′-CCAGAGAATGTCAGC-AGTGC-3′) from genomic DNA (AB, India and Tü strains), and from maternal-specific cDNA generated from wild-type, *sur*$^{m768/m768}$ and *sur*$^{ty68b/+}$ oocytes (Superscripts, Gibco). The amplified DNA was cloned in pGEMT-Easy (Promega) and sequenced (Lycor, Amersham, NIF1236) using SP6, T7 and internal primers.

### *RNA injection*

The coding region of wild-type, *sur*$^{m768}$ and *sur*$^{ty68b}$ mutant *foxH1* cDNA were cloned in pCS2+ [63]. To generate the fusion constructs, the SID-encoding sequence of wild-type and both mutant FoxH–pCS2+ constructs was replaced (*Stu*I–*Xba*I) with cDNA encoding GFP, the repressor domain of *Drosophila* Engrailed or the activator domain of VP16. The *engrailed* and *VP16* cDNA were modified by PCR using primers: en5-RV (5′-GATATCGCCCTGGAGGATCGC-3′), en3-Xba (5′-TCTAGAGAGCAGATTTCTCTGG-3′), VP5-RV (5′-GATATCAC-CGCCCCCATTACC-3′) and VP3-Xba (5′-TCTAGACACCGTACT-CGTCAAT-3′). Sense RNA for injection was generated from *Acc*651-linearized FoxH1 or Fkh fusion pCS2+ constructs using SP6 mMassage mMachine Kit (Ambion). The *cyc* and *sqt* RNA was prepared as described [4,64] and quantified parallel to RNA standards by agarose gel electrophoresis. About 1 nl diluted RNA (1–250 ng/μl in 0.1 M KCl, as indicated for the various experiments) was injected through the chorion of 1-cell or 2-cell stage embryos.

### In situ *hybridization*

*In situ* hybridization was performed as described [65]. The *foxH1* antisense RNA was synthesized from *Cla*l-digested *foxH1*-pCS2+ plasmid, using T7 RNA polymerase. Other riboprobes were generated as described (see text).

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